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Rossbeevera yunnanensis (*Boletaceae, Boletales*), a new sequestrate species from southern China

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Abstract — A new sequestrate (truffle-like) species, *Rossbeevera yunnanensis,* is described based on a collection from Chuxiong Prefecture, Yunnan Province, China. The species is morphologically characterized by its large, narrow, fusoid to fusiform basidiospores and remarkably thin peridium. Maximum likelihood and neighbor joining phylogenies of our nLSU rDNA dataset indicate that the species constitutes the earliest diverging lineage within the genus *Rossbeevera* and has a close phylogenetic relationship to species of *Leccinellum.* Morphological and phylogenetic relationships to the other Asian members of *Rossbeevera* are discussed.

Key words — *Chamonixia,* FTA, hypogeous fungi, *Leccinum*, *Rosbeeva*

Introduction

The sequestrate (truffle-like) genus *Rossbeevera* T. Lebel & Orihara (erroneous orthographic variant: "*Rosbeeva*") was erected to accommodate Australasian and Asian species of *Chamonixia* Rolland (Lebel et al. 2012a,b), which are phylogenetically close to but distinct from *Chamonixia* sensu stricto within *Boletaceae*. The genus is characterized by ellipsoid to fusiform basidiospores with 3–5 longitudinal ridges, bluish green to cyanescent discoloration of basidiomata, and a thin cutis-like whitish peridium. Phylogenetically, this genus is closely related to the epigeous bolete genera *Leccinum* Gray and *Leccinellum* Bresinsky & Manfr. Binder and sequestrate genera *Chamonixia* and *Octaviania* Vittad. (Lebel et al. 2012a, Orihara et al. 2012). *Rossbeevera* species have

been previously reported from Australia—*R. vittatispora* (G.W. Beaton et al.) T. Lebel and *R. westraliensis* T. Lebel, New Zealand—*R. pachydermis* (Zeller & C.W. Dodge) T. Lebel, Southeast Asia—*R. mucosa* (Petri) T. Lebel, China—*R. bispora* (B.C. Zhang & Y.N. Yu) T. Lebel & Orihara, and Japan—*R. eucyanea* Orihara and *R. griseovelutina* Orihara (Lebel et al. 2012a,b).

During a fieldtrip in Yunnan Province, China, two of the authors (Smith & Ge) collected *Rossbeevera* basidiomata that could not be ascribed to any currently known species. Here we propose this material as a new species, *Rossbeevera yunnanensis*, based on morphological evidence as well as phylogenetic analyses of the large subunit of the nuclear ribosomal DNA (nLSU).

Materials & methods

Taxon sampling and macro- and microscopic characterization

Fresh basidiomata were located and collected using a truffle rake. Specimens were placed in wax paper bags and transported to the laboratory within six hours where they were photographed and rapidly dried in a closed container with silica drying beads. The dried specimens are deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (KUN, with HKAS accession numbers), Kanagawa Prefectural Museum of Natural History (KPM), and the University of Florida Herbarium at the Florida Museum of Natural History (FLAS).

For standard light microscopy and differential interference contrast microscopy, hand-cut sections of dried specimens were mounted in water, 3% KOH, lacto-glycerol, or 1% phloxine B aqueous solution. To determine the amyloid reaction, dried material was stained with Melzer's reagent. Spore dimensions (length × width range) and their standard deviations (SD), and length of hilar appendages were measured based on 50 randomly selected spores. The length : width ratio (*Q*) and hilar appendage : spore length ratio (*HA/S*, newly introduced here) are presented to describe a range of the basidiospore proportion. Measurements include the hilar appendage but not ornamentation or pedicel. Basidium ranges are given as length range × width.

Molecular methods

DNA was extracted from a dried basidioma using the Indicating FTA Cards (Whatman International Ltd, Maidstone, England). Internal tissue ca. 1 mm thick was soaked in 99.5% ethanol for 2 min before DNA extraction. The extraction was done according to the manufacturer's protocol for plant samples (available at http://www.whatman.com/References/WGI_1397_PlantPoster_V6.pdf) using FTA Cards, more convenient and efficient over traditional methods (Dentinger et al. 2010). Furthermore, we demonstrate here that this technique is useful for dried tissues after a brief ethanol pretreatment. For PCR amplification and sequencing reactions, we used the primer pair LR0R / LR5 (Vilgalys & Hester 1990). For PCR conditions, sequencing and editing of the obtained sequence, see Lebel et al. (2012a). The nLSU sequence is deposited in GenBank.

Sequences included in the nLSU dataset were retrieved from GenBank based on the previous analyses of *Rossbeevera* and allied genera (Lebel et al. 2012a). Some sequences

of *Leccinellum* spp., which are shown to be paraphyletic with *Rossbeevera*, were selected according to the phylogeny in Lebel et al. (2012a) and were used to root the phylogenetic tree. Multiple sequence alignment was performed using Clustal X ver. 1.83 (Thompson et al. 1997) and the data were manually adjusted in SeaView (Galtier et al. 1996). Gaps were treated as "missing" data for all analyses.

The neighbor-joining (NJ) analysis based on p distance was conducted with Clustal X ver. 1.83 with 1000 bootstrap replicates and with default settings. Subsequently, the maximum likelihood (ML) analysis was done by PhyML ver. 3.0 (Guindon et al. 2010) under GTR + I + G model based on hLRT estimated by MrModeltest 2.3 (Nylander 2004), starting the analysis using a tree estimated by the Nearest Neighbor Interchanges (NNIs) and setting the number of bootstrap replicates to 1000. The resulting trees were visualized with FigTree ver. 1.3.1 (Rambaut, available at http://tree.bio.ed.ac.uk/).

Results

The finally aligned nLSU dataset was 837 bp long, including full length reads for all 19 sequences but excluding the primer regions. Settings of the best model estimated by hLRT in MrModeltest 2.3 were as follows: base frequencies (A = 0.2700, C = 0.1941, G = 0.2904, T = 0.2455), proportion of invariable sites (I = 0.7687), and gamma distribution shape parameter (a = 1.9606). Maximum likelihood analysis generated one ML tree (ln L = –2078.576887; Fig. 1). Branches that were moderately to strongly supported by ML bootstrap values (>60%) were also strongly supported in the NJ topology (BS >70%). In the *Rossbeevera* clade, *R. eucyanea* and *R. griseovelutina* were each supported to be monophyletic by high BS values but the Australasian species were not well differentiated from each other, as shown in Lebel et al. (2012a). The Chinese *Rossbeevera* sp. MES420 formed a basal branch within the *Rossbeevera* clade and was phylogenetically distinct from the other members of *Rossbeevera* included in this study.

Taxonomy

Rossbeevera yunnanensis Orihara & M.E. Sm., **sp. nov.** Fig. 2

MycoBank MB 563649

Differs from *Rossbeevera bispora* by thinner or partially lacking peridium, fusoid to fusiform basidiospores, and 2–4-spored basidia.

Type: China, Yunnan Province, ChuXiong Yi Autonomous Prefecture, Mt. Zixi, 19 Sept. 2010, M. E. Smith et Z.-W. Ge, MES420 (**holotype** - HKAS 70689, **isotype** - KPM-NC0017850, GenBank JN979437).

Etymology: *yunnanensis* (Latin) refers to the type locality.

Basidiomata sparse, ca. 1 cm, subglobose, soft, surface covered with very thin, minutely felty, whitish to almost translucent peridium gradually turning bluish gray or black when touched or bruised, the peridium partially absent, exposing the gleba. Gleba off-white to beige when immature, reddish brown to blackish

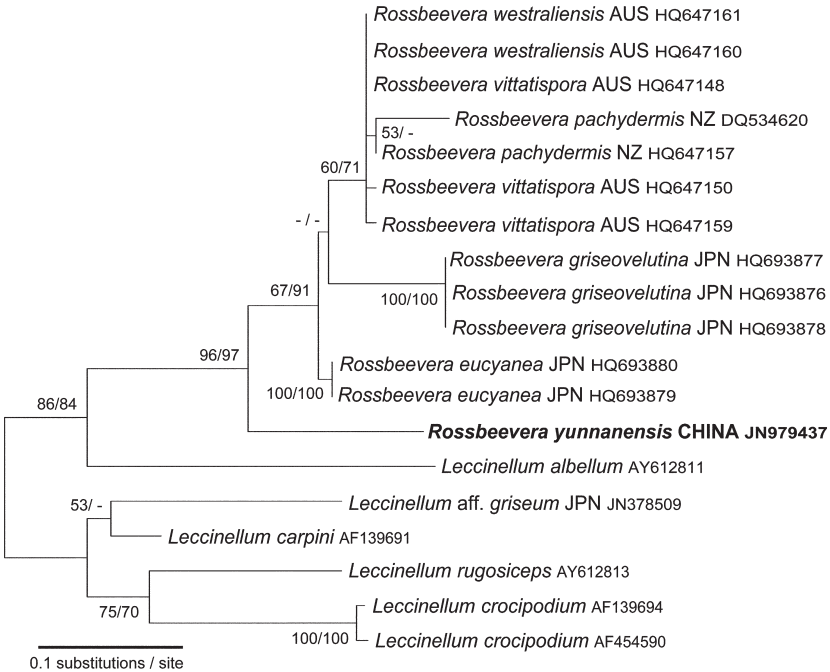

Figure 1. Maximum likelihood (ML) tree of the nLSU dataset of *Rossbeevera* species with *Leccinellum* species selected as the outgroup. ML and Neighbor-Joining (NJ) bootstrap (BS) values (1000 replicates; only BS >50% are shown) are indicated above or below branches or at nodes as MLBS/NJBS. Abbreviations: AUS = Australia; NZ = New Zealand; JPN = Japan.

brown at maturity, rubbery, composed of minute, irregular locules, turning bluish gray in some portions when cut and exposed to air, trama subgelatinous, somewhat translucent. Stipe-columella present but reduced, somewhat dendroid or as a small basal pad, subgelatinous, translucent. Odor unknown.

Basidiospores (14.4–)16.9–23.4(–24) × 6.8–9(–9.5) µm, (mean ± SD = 20.1 ± 1.60 × 7.8 ± 0.61), *Q* = 2–3.8 (mean = 3.1), symmetric, fusoid to fusiform, inamyloid, nondextrinoid, colorless at first then becoming reddish brown at maturity, with 3–4 or rarely 5 smooth, longitudinal ridges up to 2.3 µm high (mostly ca. 1 µm high) in water, walls 0.7–1.5 µm thick, with a large hilar appendage (2–)2.3–3.8 µm long (mean 3.1), HA/S = 0.12–0.22, (mean = 0.16) at the base and a minute, incomplete hollow (> 1 µm in diameter) at the tip. Basidia 17.5–30 × 6.8–11 µm (mean = 23.5 × 8.4 µm; n = 15), cylindrical to cylindro-clavate, colorless to pale yellow-brown to reddish brown, 2-, 3- or

4-spored. Hymenium developed when immature but collapsed at maturity, colorless, hymenial cystidia not seen; basidioles cylindrical or clavate to clavulate. Subhymenium not developed. Trama of densely or somewhat loosely interwoven, partly branched, colorless, non-inflated, thin-walled (< 0.8 µm thick), filamentous hyphae 3.5–8 µm broad. Stipe-columella composed of loosely interwoven, subgelatinous, thin-walled (< ca. 1 µm thick) filamentous hyphae (2–)5–8(–9.5) µm broad. Peridium thin, absent in some portions, up to 60 µm thick in the dried basidioma, yellowish brown under light microscopy, composed of repent, non-inflated, thin-walled (< 0.8 µm thick) filamentous hyphae 2.5–6 µm broad but the hyphae are collapsed in most portions. Clamp connections absent in all tissues.

Habitat and distribution: Hypogeous in mixed forest dominated by *Pinus yunnanensis* Franch., *Lithocarpus mairei* (Schottky) Rehder, *L. dealbatus* (Hook. f. & Thomson ex Miq.) Rehder, and unidentified *Quercus* spp.; known only from Yunnan Province, China.

Discussion

Rossbeevera yunnanensis shares several morphological characteristics with the other *Rossbeevera* species, including the bluish color change of the peridium and reddish brown basidiospores with 3–5 unbranched longitudinal ridges. This new species also has the large, distinctly fusoid to fusiform (Q = 2.0–3.8) basidiospores and remarkably thin peridium characteristic of the genus. *Rossbeevera griseovelutina*, which is reported from Japan, has slightly larger basidiospores (14.4–31.9 × 6.7–10.4 µm, mean = 22.2 × 8.7 µm) and the Q value is smaller (Q = 2.0–3.3; Lebel et al. 2012a). In addition, the latter species has a more fully developed peridiopellis composed of more or less vertically oriented, partially inflated hyphae. *Rossbeevera yunnanensis* also differs morphologically from another known Chinese species, *R. bispora*, because *R. yunnanensis* has a thinner peridium, larger and narrower basidiospores, and its basidia often bear 4 spores rather than 2 spores characteristic of *R. bispora* (Zhang & Yu 1989). We have examined the holotype of *R. bispora* (GDGM5688) and have confirmed the above-noted differences. The Southeast Asian species, *R. mucosa* is readily distinguished from *R. yunnanensis* in its ellipsoid to fusoid basidiospores (13–)15–17 µm in diameter with a lower Q value (Q = 1.76–2.05; Lebel et al. 2012a).

The HA/S ratio, which is newly introduced in this study, could be helpful to describe spore shapes objectively when spore sizes vary widely within collections or sporocarps of a particular species. Basidiospores of *R. griseovelutina* are somewhat similar to those of *R. yunnanensis* as discussed above, but the mean HA/S ratio of *R. griseovelutina* was significantly higher than that of *R. yunnanensis*. However, the ranges overlapped: HA/S of *R. griseovelutina* = 0.12–0.24, mean 0.18, n = 25 vs. HA/S of *R. yunnanensis* = 0.12–0.22, mean

= 0.16. Basidiospore morphology is widely used as a key character for the taxonomy of sequestrate fungi (Castellano et al. 1989). The HA/S difference is proposed as a new, useful diagnostic character that could be useful in the taxonomy of *Rossbeevera* and other sequestrate genera.

In our phylogeny *R. yunnanensis* formed the earliest diverging lineage within the *Rossbeevera* clade with moderate to strong bootstrap support (ML: 67%; NJ: 91%). Although we have not been able to include sequences of *R. bispora*, it is possible that the two Chinese species are closely related within *Rossbeevera*, because they share a similar overall morphology, including a cutis-like peridium and relatively large basidiospores with mostly 3–4 longitudinal ridges. Sequences from additional genes and specimens of *Rossbeevera* from China and the adjacent regions would help to clarify the evolutionary origin of this sequestrate genus.

Although we have examined only one *R. yunnanensis* collection, its morphological characteristics and unique phylogenetic position are sufficient to discriminate the species from the other members of *Rossbeevera*. We would prefer to have additional specimens in order to provide more ecological information about *R. yunnanensis*, such as details of its phenology and host tree preferences. However, since hypogeous fungi are difficult to find and collect, we felt it was important to describe this new *Rossbeevera* species to promote further research on the systematics of Asian sequestrate fungi. Unique sequestrate fungi have been found in Asia for more than a century (e.g. Corner & Hawker 1953; Smith & Schmull 2011). However, several recent studies highlight the fact that Asia is likely a hotspot for unique, endemic truffles and probably hosts a high diversity of undescribed sequestrate fungi (Yang et al. 2006; Desjardin et al. 2008, 2009; Orihara et al. 2008, 2010, 2012; Kinoshita et al. 2011). Clearly, more taxonomic work on sequestrate fungi is needed in this region.

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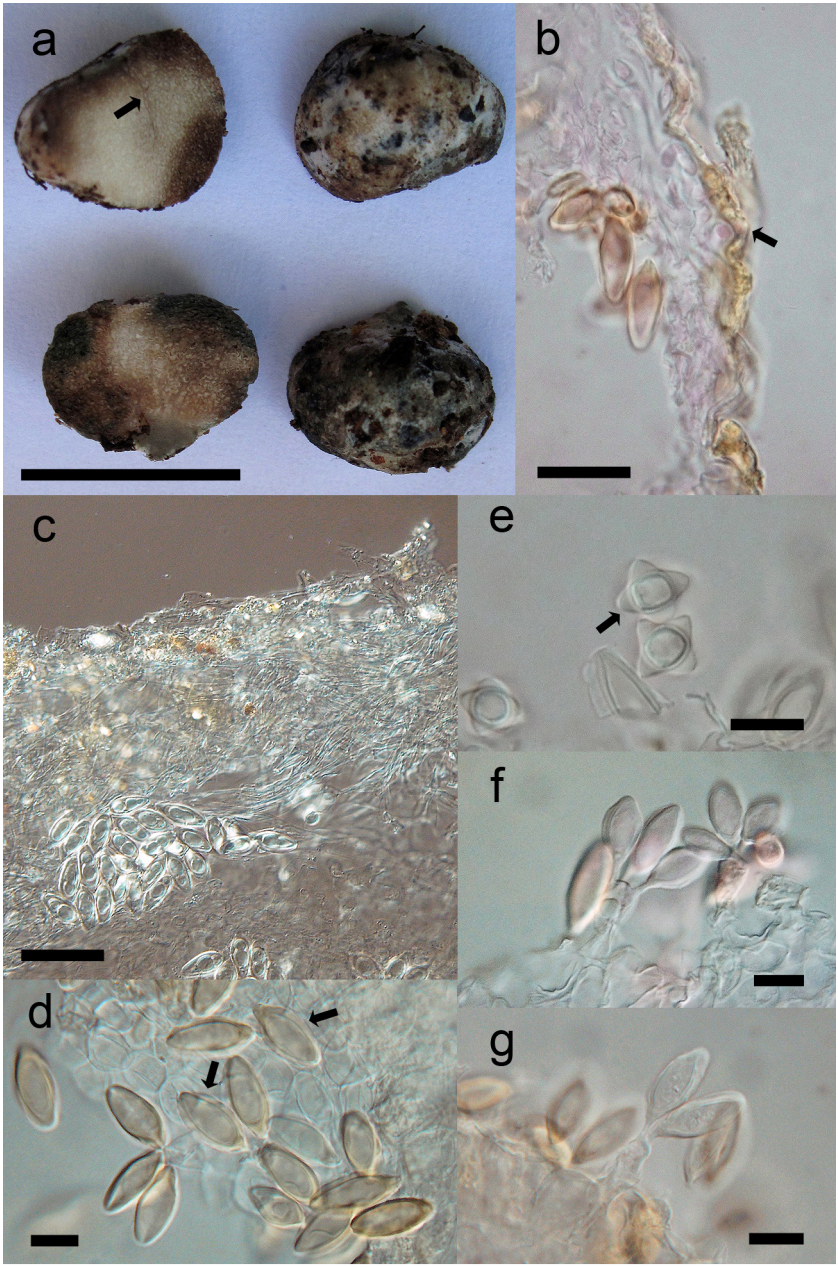
Figure 2 (right). *Rossbeevera yunnanensis* (holotype): a. Basidiomata. A reduced columella is present in the center of the section (arrow); b. Significantly reduced peridium of dried material (arrow); c. Thick portion of the peridium; d. Mature basidiospores with longitudinal ridges (arrows) mounted in lacto-glycerol; e. Immature basidiospores viewed from above (arrow); f. 3- and 4-spored basidia mounted in lacto-glycerol after a pretreatment with 1% phloxine B aqueous solution; g. 2-spored basidium mounted in lacto-glycerol. Scale bars: a = 1 cm; b = 20 μm; c = 30 μm; d–g = 10 μm.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